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Detection of Tn5-Like Sequences in Kanamycin-Resistant Stream Bacteria and Environmental DNA

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Resistance to kanamycin and neomycin in the bacterial assemblage of a coastal plain stream was detected by growth of colonies on media containing antibiotics. Three of 184 kanamycin-resistant colonies hybridized with a probe containing the *nptII* gene from transposon Tn*5*; the *nptII* gene encodes the enzyme neomycin phosphotransferase and conveys resistance to kanamycin and neomycin. In one of these isolates, the homologous gene was cloned and shown to confer resistance to a kanamycin-sensitive *Escherichia coli* strain. Since enumeration of bacteria by acridine orange direct counts revealed that less than 0.2% of the bacteria present were cultivated, direct examination of environmental DNA was used to assess abundance of sequences that hybridize to the *nptII* gene. To examine the resistance potential of bacteria that were not cultured, total DNA was extracted from environmental samples and hybridized with specific probes. The relative amount of eubacterial DNA in each sample was determined by using a eubacterial specific rDNA probe. Then, the abundance of sequences that hybridize to the eubacterial neomycin phosphotransferase gene was determined by hybridization and expressed relative to the total eubacterial DNA in the assemblage. Relative gene abundance was significantly different among assemblages from different habitats (leaves, midchannel sediments, and bank sediments) but did not differ among stream sites.

Studies of antibiotic resistance in bacteria from environmental samples have relied on culturing to document resistance frequency (11, 13) or have probed cultivated bacteria for the presence of particular antibiotic resistance genes (16). In both cases, only the culturable portion of the bacterial assemblages was examined. Since culturable bacteria are often a minor component of the total bacterial assemblage, relying on culture of environmental bacteria to describe features of assemblages has been questioned (19).

Techniques developed to describe nonculturable bacteria commonly involve extraction and examination of nucleic acids from environmental samples (10, 14, 17, 28, 30, 31). Abundances of a few specific bacterial genes have been determined by hybridization of gene probes to environmental DNA (3, 10, 18, 26, 27, 32). Although direct examination of DNA allows one to circumvent the problems associated with low culturability of environmental bacteria, examination of environmental DNA presents a new set of difficulties. First, detection of a specific DNA sequence by hybridization does not demonstrate that the gene is functional, nor does it indicate the level to which the gene is transcriptionally active. Second, genes with widely different nucleotide sequences may encode enzymes conferring similar phenotypes, further obscuring the relationship between gene abundance and functional potential. Evaluation of the usefulness of any probe must address these issues.

In this study we report the occurrence of kanamycin and neomycin resistance in the culturable portion of the bacterial assemblage of a South Carolina stream. To provide information on kanamycin resistance potential in the total bacterial assemblage, DNA extracted from environmental samples was examined for the presence of the neomycin phosphotransferase (*nptII*) gene, which conveys resistance to kanamycin and neomycin (12). Although there are several genes that confer kanamycin resistance (4, 7), the constitutively expressed *nptII* gene was chosen because it is located on transposon Tn*5*, which has a well-developed physical and genetic map and is widely distributed among the eubacteria (12, 15). Preliminary hybridization studies indicated the presence of *nptII*-like genes in DNA extracted from stream sediments. To examine the adequacy of the *nptII* probe in describing kanamycin resistance potential of the total bacterial assemblage, we determined whether functional copies of the *nptII* gene were found in kanamycin-resistant, culturable stream bacteria. We assessed the abundance of *nptII* in kanamycin-resistant isolates to determine whether this single gene accounts for a significant fraction of the observed resistance. Having evaluated the utility of this approach to kanamycin resistance potential, we determined whether there were spatial differences in the relative abundances of *nptII* in samples taken from different locations and habitats in a stream.

MATERIALS AND METHODS

Samples were collected from three sites in the Upper Three Runs Creek (UTR) drainage on the U.S. Department of Energy's Savannah River Site in Aiken, S.C. One site on Tinker Creek (Tin), a tributary of UTR, and two sites on the UTR main stem (UTR 1 and UTR 2) were sampled. Tin was approximately 7 km upstream from UTR 1. UTR 1 was approximately 5 km upstream from UTR 2. Five 200-ml water samples were collected from each site on 17 September 1990. Medium containing kanamycin or neomycin, nutrient broth (2 g liter^{-1} [Difco] 25% of normal strength) in a minimal salts solution (in milligrams per liter: NaCl, 2.5; $MgSO_4$, 0.1; $CaCl_2$, 0.01; Na_2HPO_4, 7.0; KH_2PO_4, 3.0; NH_4Cl, 1.0), and 1.5% agar (Difco) was inoculated with 0.5 ml of sample. Antibiotics were added to autoclaved medium

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in filter-sterilized solutions to final concentrations of 50 and 200 μg ml^{-1}, respectively. Cycloheximide (100 μg ml^{-1}) was also added to inhibit fungal growth. Plates containing only cycloheximide were used to determine the abundance of culturable bacteria and were inoculated with 0.01 and 0.1 ml of sample. After incubation at room temperature for 48 h, colonies were enumerated. Total bacterial densities were determined from three samples per site by acridine orange direct counts (9).

To examine the potential basis of the observed resistance, 184 kanamycin-resistant and 300 kanamycin-susceptible (200 μg of kanamycin ml^{-1}) isolates were hybridized to the 1.0-kbp *Bgl*II-*Sal*I restriction fragment from transposon Tn*5* by a modified version of the Grunstein and Hogness colony hybridization procedure (8, 23). This fragment contains the entire coding region for *nptII* (25). Although there are several mechanisms for kanamycin resistance, this gene was chosen because Tn*5* has a well-developed physical and genetic map and a wide phylogenetic distribution among eubacteria (12, 15). Colonies attached to Whatmann 3MM paper were lysed with 0.5 M NaOH, neutralized in 1 M Tris (pH 7.5), and immersed in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and then 95% ethanol. Filters were dried at 32°C for 2 h (23). Colonies were hybridized to probe DNA labeled with digoxigenin-dUTP by random-primer extension (6). Hybridizations were performed under stringent conditions with 5× SSC, 0.5% blocking reagent, 0.1% sodium *N*-lauroyl sarcosine, and 0.02% sodium dodecyl sulfate (SDS) at 68°C for 12 to 18 h as recommended by Boehringer Mannheim for the Genius nonradioactive DNA labeling and detection kit. Washes were performed four times with 0.1× SSC–0.1% SDS for 20 min at 68°C. Hybridization results were visualized by using a Lumi-Phos system as recommended by the manufacturer (Boehringer Mannheim).

Total genomic DNA was extracted and purified (33) from the three positive colonies that hybridized the Tn*5* probe and from one negative colony. DNAs from the isolates and appropriate controls were digested with the restriction enzyme *Hin*dIII, which generates a unique 3.3-kbp restriction fragment internal to Tn*5* (12). The negative control DNA was extracted from *Myxococcus xanthus* DK1622, which does not contain a Tn*5* insertion (24). Genomic DNA from *M. xanthus* DK1985, which contains a single Tn*5* insertion, served as a positive control. DNA fragments were separated on a 0.7% agarose gel, transferred to Hybond-N nylon (Amersham), and hybridized to the digoxigenin-dUTP-labelled Tn*5* DNA fragment as described above.

To determine whether the kanamycin resistance expressed by the three colonies that hybridized the Tn*5* probe was attributable to the hybridizing fragment, *Hin*dIII-digested DNAs from the colonies were ligated with *Hin*dIII-digested pUC19. *Escherichia coli* JM83 was electrotransformed with the ligation products (5). Transformants were selected for resistance to 80 μg of kanamycin ml^{-1} and 50 μg of ampicillin ml^{-1} (vector encoded) on LB agar (21). Plasmids were isolated from transformants with a Magic Mini-Prep kit (Promega, Madison, Wis.) and digested with *Hin*dIII. The molecular sizes of the restriction fragments were estimated by comparison with known standards on an agarose gel.

Total environmental DNA was extracted as described by Ogram et al. (17) from leaves and sediment samples collected at the three sites. Initially, in October 1990, one sediment and one leaf sample were collected from each site. In December 1990, three replicate samples were collected at each site from leaves, midchannel sediments, and bank sediments (total of 27 samples). Approximately 50 g (wet weight) of leaves, 100 g of midchannel sediments, and 30 g of bank sediments were required to give sufficient amounts of eubacterial DNA. Although extracted DNA solutions were brown because of contamination with humic material, the DNA content of diluted samples was roughly quantified on DNA Dipsticks (Invitrogen) by comparing environmental DNA with known quantities of standard DNA. Environmental DNA was blotted onto Hybond-N nylon with a Mini-Fold II slot blot apparatus (Schleicher and Schuell). The eubacterium-specific oligonucleotide probe 5′-GCTGCCTCCCGTAG GAGT-3′, which hybridizes to positions 338 to 355 of the 16S rRNA gene of all 250 eubacteria for which complete and partial 16S rRNA sequences are available (1), was end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (21). The optimal hybridization conditions used with the oligonucleotide probe were determined empirically by using kingdom-specific DNA samples with *Methanococcus maripaludis* DNA as the archaebacterial representative, salmon sperm DNA as the eukaryotic representative, and *M. xanthus* DK1622 as the eubacterial representative. Hybridization conditions were considered optimal when the eubacterium-specific oligonucleotide probe hybridized to the eubacterial standard but did not hybridize to the archaebacterial and eukaryotic DNAs. Filters were prehybridized in 6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7])–1% SDS–10× Denhardt's solution–100 μg of denatured salmon sperm DNA or yeast tRNA per ml at 55°C for 2 h, hybridized in 6× SSPE–1% SDS at 55°C overnight, washed three times for 8 min each at room temperature in 6× SSPE–1% SDS, and washed once at 55°C in 1× SSPE–1% SDS for 2 min. The radiolabelled oligonucleotide probe was removed by incubating the nylon filter in 5 mM Tris-HCl (pH 8.0)–0.2 mM EDTA–0.05% tetrasodium pyrophosphate–0.1× Denhardt's solution for 2 h at 73°C. The conditions used when hybridizing the digoxigenin-dUTP-labelled kanamycin resistance marker of Tn*5* to environmental DNA were as described above for colony hybridizations.

The eubacterium-specific oligonucleotide probe was hybridized with 0.0625, 0.25, and 1 μg of total DNA from each environmental sample. Densitometry was used to normalize hybridization signals to the *M. xanthus* DK1985 standard, whose signal strength was arbitrarily assigned a value of 1. The nylon filter was then stripped of the probe and hybridized with the 1.0-kbp *Bgl*II-*Sal*I fragment of Tn*5* containing the *nptII* gene. The densitometrically determined hybridization signal strength of *M. xanthus*, which contains a single Tn*5* insertion, was arbitrarily assigned a value of 100. Hybridization signal strengths of the samples were again normalized to *M. xanthus* and divided by the relative amount of eubacterial DNA. As the incidence of false-positive signals among cultivated bacteria was zero, we assumed that the intensity of hybridization was related to the abundance of the *nptII* gene in the sample and refer to the calculated values as relative *nptII* gene abundance.

Differences among sampling sites were compared by using analysis of variance. For significant differences, P was ≤ 0.05. Analyses were performed with a Statview 512+ program (Brainpower, Inc.) and an Apple Macintosh computer.

RESULTS

Bacteria collected from the water column at all three sites grew on media containing kanamycin or neomycin at 50 and 200 μg ml^{-1} (Fig. 1). There were statistically significant differences among sites in the numbers of resistant bacteria (for kanamycin: site effect, $F = 15.30$ and $P < 0.01$;

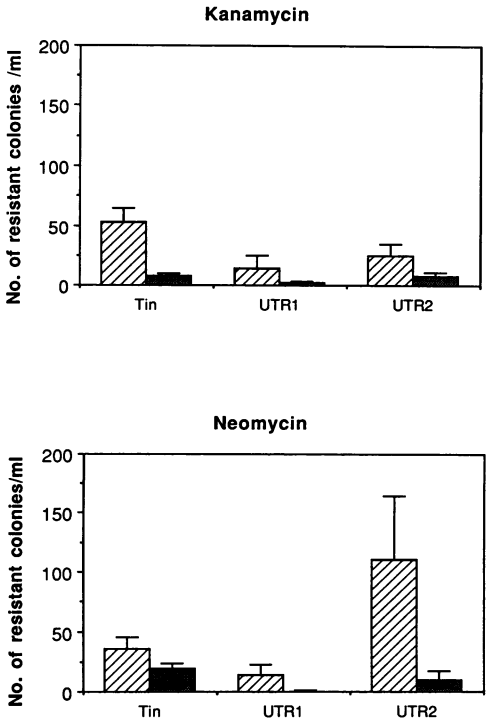

FIG. 1. Resistance to kanamycin and neomycin in water column bacteria. Values are means of five replicates and standard errors. Concentrations of antibiotics: ▨, 50 μg ml^{-1}; ▩, 200 μg ml^{-1}.

antibiotic concentration effect, $F = 5.49$ and $P = 0.02$; concentration by site interaction, $F = 2.75$ and $P = 0.09$) (for neomycin: site effect, $F = 7.14$ and $P = 0.01$; antibiotic concentration effect, $F = 3.48$ and $P = 0.05$; concentration by site interaction, $F = 2.90$ and $P = 0.08$). The sites did not differ significantly in the abundances of total bacteria (from acridine orange direct counts) and culturable bacteria (Table 1; $F = 4.24$ and $P = 0.07$ and $F = 0.02$ and $P = 0.98$, respectively). At all sites, less than 0.2% of the bacteria observed microscopically were cultivated.

Kanamycin-resistant and -susceptible colonies were probed for the presence of the *nptII* gene by colony hybridization. Of the 184 kanamycin-resistant colonies examined, 3 hybridized with the probe. None of the 300 kanamycin-susceptible colonies hybridized with the probe under stringent hybridization conditions. Isolates that hybridized with

TABLE 1. Abundances of bacteria in water

Site	Total abundance[a] (no. of bacteria ml^{-1}, 10^5)	CFU[b] (no. of CFU ml^{-1}, 10^2)	% Culturable[c]
Tin	2.74 (0.26)	3.44 (0.65)	0.13
UTR1	4.81 (0.31)	3.14 (2.16)	0.06
UTR2	4.11 (0.48)	3.05 (0.64)	0.07

[a] Total bacterial abundances obtained from direct counts of acridine orange-stained cells. Numbers are means of three samples with standard errors within parentheses.

[b] Abundance of CFU determined from culturing on nutrient agar as described in Materials and Methods. Numbers are means of five samples with standard errors within parentheses.

[c] Abundances from CFU divided by total bacterial abundance from microscopic counts multiplied by 100.

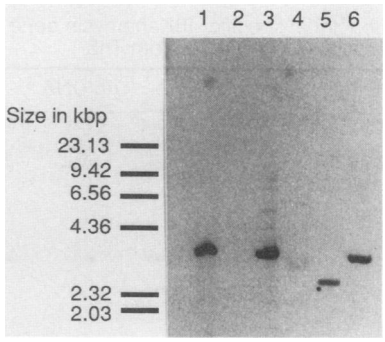

FIG. 2. Southern blot analysis of genomic DNA from UTR2 isolates resistant to 200 μg of kanamycin ml^{-1} with the *Bgl*II-*Sal*I 1.0-kbp fragment from Tn*5* as a probe. DNA was digested with *Hin*dIII (which results in a 3.0-kbp internal fragment in transposon Tn*5*), and 5 μg was applied. Lanes: 1, *M. xanthus* DK1985; 2, *M. xanthus* DK1622; 3, 5, and 6, isolates that hybridized the *Bgl*II-*Sal*I 1.0-kbp probe from Tn*5* in colony blot analysis; 4, isolate that did not hybridize the *Bgl*II-*Sal*I Tn*5* fragment in colony blot analysis.

the probe and one isolate that did not hybridize were analyzed by Southern blotting with a probe containing the *nptII* gene. *Hin*dIII-digested DNA from *M. xanthus* DK1985, a strain known to harbor a single Tn*5* insertion, contained a 3.3-kbp fragment that was homologous with the probe, whereas DNA from *M. xanthus* DK1622, which lacks this transposon, did not show homology with the probe (Fig. 2, lanes 1 and 2). Two of the positive isolates contained a homologous band similar in size to that of *M. xanthus* DK1985 (lanes 3 and 6), suggesting that their resistance was due to the *nptII* gene of Tn*5*. The remaining positive isolate contained a 2.5-kbp fragment homologous to Tn*5* (lane 5). The isolate that did not hybridize to the probe in the colony blot also failed to hybridize to the probe in the Southern blot (lane 4).

DNA from one of the kanamycin-resistant isolates that hybridized the *nptII* probe was ligated with a plasmid vector (pUC19) to determine whether kanamycin resistance could be transferred to a nonresistant strain (*E. coli* JM83). After transformation of *E. coli* JM83 with DNA from isolate 1 (Fig. 2, lane 1), plasmids from 3 of 12 transformants were digested with *Hin*dIII; the inserted fragment was approximately the same size as that observed on the Southern blot.

Since only a small portion of the bacterial cells observed in environmental samples could be cultivated, it was of interest to examine both culturable and nonculturable bacteria by extraction of total DNA from environmental samples. Hybridization of environmental DNA with kingdom-specific probes for eubacteria, archaebacteria, and eukaryotes revealed that samples contained DNA from all three kingdoms (data not shown). The environmental DNA samples were hybridized to the eubacterium-specific probe to determine the relative abundance of eubacterial DNA in each sample. Results from samples collected in October 1990 are shown in Fig. 3. Densitometry was used to normalize hybridization signals to the *M. xanthus* standard (Fig. 3A). The nylon filter was then stripped of the probe and hybridized with the 1.0-kbp *Bgl*II-*Sal*I fragment of Tn*5* (Fig. 3B). Comparison of signal strengths indicates wide variation in abundance of sequences homologous to the *nptII* gene among the eubacterial DNA. For example, samples in lanes 6, 7, and 8 show strong hybridization with the eubacterial kingdom-specific probe but low hybridization with the *nptII* gene probe,

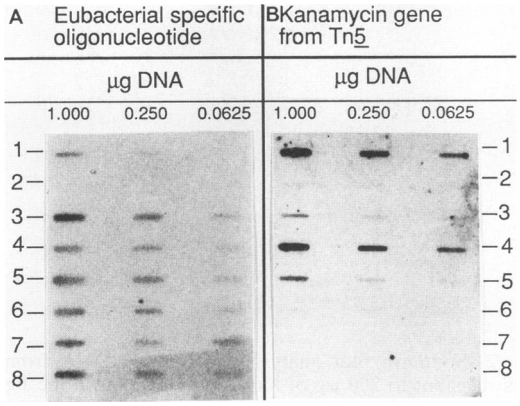

FIG. 3. Slot blot analysis of environmental DNA with the eubacterium-specific oligonucleotide (A) and the *Bgl*II-*Sal*I 1.0-kbp fragment from Tn*5* (B) as probes. Numbers at left and right indicate the following DNA samples: 1, *M. xanthus* DK1985; 2, salmon sperm; 3, UTR1 bank sediments; 4, UTR2 bank sediments; 5, Tin bank sediments; 6, UTR1 leaves; 7, UTR2 leaves; 8, Tin leaves. The amounts of total DNA per well are indicated above each lane.

indicating a low abundance of sequences homologous to this gene in the sample. In contrast, the sample in lane 4 hybridized strongly with both probes, indicating that *nptII*-like sequences were abundant in this sample.

In a larger sampling effort in December 1990, the relative abundance of eubacterial DNA and the *nptII*-like sequences was determined from three samples collected from each of three sites and three habitats. The values presented in Fig. 4 are assumed to reflect the relative abundance of the *nptII* gene among the eubacterial component of the environmental samples. The relative abundances of the *nptII* gene were significantly different among habitats, with leaves having lower abundances than bank sediments (Fig. 4; $F = 5.04$, $P = 0.02$). However, there were no significant differences in the abundances of this gene among the three sites examined ($F = 0.30$, $P = 0.74$).

DISCUSSION

Although antibiotic resistance has been compared in assemblages of native aquatic bacteria (11, 13), conclusions concerning the distribution of antibiotic resistance may be compromised by the low percentage of bacteria that can be cultivated from such environments. Because less than 0.2% of the bacteria from our study stream were cultivated, we assessed the frequency of an antibiotic resistance gene in the total bacterial assemblage by examining DNA prepared from environmental samples. The method of DNA extraction used in this study involved grinding environmental samples with glass beads (17) and was selected because it effectively disrupts the most impermeable cells and spores (29). As this technique results in sheared DNA, which is unsuitable for Southern blotting, the presence of the *nptII* gene, which confers kanamycin resistance, was assessed by hybridization of slot blotted DNA under relatively stringent conditions. Significant hybridization signals were found in most samples, prompting a closer inspection of the significance of the hybridization results.

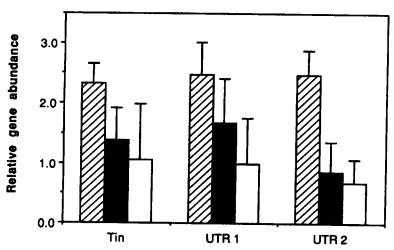

FIG. 4. Relative abundance of the *nptII* gene in environmental samples. Values are means of three replicates and standard errors. Bars: ▨, bank sediments; ▩, midchannel sediments; ▭, leaves.

To determine whether the probe accurately identifies *nptII*-like genes, colonies of culturable stream bacteria were examined for the ability to hybridize the probe. None of the 300 kanamycin-sensitive isolates tested hybridized with the *nptII* gene, suggesting that cross-hybridization with nonfunctional genes is rare. Two of the three kanamycin-resistant isolates that hybridized with the probe contained a restriction fragment similar in size to that predicted from the physical map of Tn*5*. In one of these strains, the *nptII*-like gene was shown to confer kanamycin resistance to *E. coli*. These results suggest that the probe accurately identifies functional *nptII* genes. Because of the strong selection for kanamycin resistance, it was possible to demonstrate that the *nptII*-like gene conferred kanamycin resistance to *E. coli*. Similar genetic selections are not available for most environmental genes. Amplification by polymerase chain reaction (20) may allow isolation of the target gene in the absence of a strong selection.

The presence of multiple mechanisms of kanamycin resistance in stream bacteria was suggested by the detection of an *nptII*-like gene in only a few kanamycin-resistant bacteria. Other genes encoding kanamycin resistance but lacking homology with the *nptII* gene from Tn*5* have been reported (2, 4, 7). These results suggest that multiple probes will probably be necessary to assess kanamycin resistance potential of stream bacteria.

The *nptII* gene has been reported to occur naturally only in eubacteria. If gene frequencies are to be expressed as a percentage of eubacteria containing the gene, the proportion of eubacteria in the sample must be known. Because each sample will probably contain different amounts of archaebacterial and eukaryotic DNA, we used kingdom-specific rDNA probes to determine the percentage of eubacterial DNA in a sample. However, because the rRNA operon is present in a wide range of copy numbers on chromosomes of different bacteria (25) and since eubacterial genome size can vary over an order of magnitude (22), the exact number of eubacterial genomes cannot be determined from hybridization data alone. Future experiments employing a more direct measurement of eubacterial cell number, for example, the use of 16S rRNA-targeted oligonucleotide probes coupled with flow cytometry or in situ hybridization, may more directly assess the eubacterial cell number (1).

The bacterial assemblage composition was examined on different spatial scales under the assumption that differences in abundances of specific genes will reflect differences in abundances of specific bacteria. We measured relative abundance of the *nptII* gene among eubacteria over three spatial scales; samples were collected at three sites in the stream and at three habitats at a single site, and three independent samples were taken from each habitat. At the largest spatial scale, there were no significant differences among sites in the abundances of the *nptII* genes. However, there were differences in *nptII* abundances among different habitats at a site,

suggesting that bacterial assemblages in these habitats have different compositions. Assemblages from leaves had fewer copies of the *nptII* gene than did assemblages from sediments. It appears then that comparisons of gene abundances among environmental samples may be useful in describing bacterial assemblages from different locations.

In conclusion, DNA hybridization appears to be a useful technique for assessing the abundance of genes in mixtures of nonculturable organisms. In such studies, it is critical to demonstrate that the probe specifically hybridizes with functional copies of the gene of interest. Because of the spatial differences observed in *nptII* abundance in this study, an effort must be made in future studies to sample the many diverse habitats in an ecosystem to obtain a representative gene frequency.

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